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Review

How the virophage compels the need to readdress the classification of microbes

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ABSTRACT

The discovery of Mimivirus opened the door to reclassifying viruses and microorganisms. Because the definition of a virus had been based on their putative small size, giant viruses have been widely neglected, as have their own viral parasites: the virophage. Current studies show that giant viruses can be found worldwide in the soil, water, animals and humans.

Their existence forces us to create new classifications, the most recent being TRUC, to accommodate the existence of four branches of microorganisms, i.e., bacteria, archaea, eukaryotes and giant viruses.

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A virus among microbes

My initial training in biology was that of a rickettsiologist, which is someone who works on the intracellular bacteria rickettsia, which has long been classified between bacteria and viruses because of its parasitic involvement (Raoult and Roux, 1997). By chance, while working on bacteria resembling Legionella that had grown in amebas, we discovered giant viruses visible under the microscope, which had been mistaken for bacteria because they had a microbial appearance (La Scola et al., 2003). This led us to revise the definition of a microbe (Raoult, 2013). The word microbe appeared in the literature after a communication from Louis Pasteur in 1878, who quoted an expression of the surgeon Sedillot, to define what we could see only through the optical microscope. One of these microbes has been crucial in the discovery of vaccination by microbial modification: *Pasteurella multocida*, the agent of fowl cholera. The microbial world was then united under one name (reviewed in Ref. [27]). Later, in 1925, Chatton distinguished among microbes between those having a nucleus (eukaryotes) and those that do not have a nucleus (prokaryotes) (Raoult, 2013). This definition was purely morphological and therefore was not consistent with subsequent definitions based on genomic analysis, such as the fact that the bacteria superphyla *Planctomycetes*, *Chlamydia*, and *Verrucomicrobia* may

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Table 1
Virus milestones.

Chamberland	1885	Filter
Pasteur	1885	Rabies virus (human)
Dimitri Ivanosky's	1892	TMW (plant)
M. Beijeink	1898	TMW (plant)
Loeffer	1898	Aphtovirus (animal)
F. D'Herelle	1917	Bacteriophage (bacteria)
E. Ruska	1931	TEM and viruses
W.M. Stamley	1935	Virus are proteins
M. Temin	1965	Retrovirus
Torsvik and Dundas	1974	Virus of Archae
Raoult et al.	2004	Virus with > DNA 1 Mb genom
La Scola et al.	2008	Virophage
Desnues et al.	2012	Transpoviron

contain numbers of intracellular formations including genetic material that morphologically resembles a nucleus (Fuerst, 2005). Consequently, the microbial world was divided into two categories: eukaryotes and prokaryotes.

In *1977*, Woese, by analyzing the ribosome, proposed to divide the microbial world into three categories (Pace, 2006). The ribosome of *methanogenic and extremophilic prokaryotes* was discovered to be closer to that of eukaryotes than to bacteria. Therefore, he created the word Archaebacteria, which later became Archaea. This term is *misleading* because it implies that those microbes were archaic prokaryotes based on the fact that the first "archaea" were found among extremophile bacteria living at very high temperatures; these prokaryotes were thought to be the origin of life. This name led researchers to believe that these prokaryotes were interesting only for environmental microbiologists, which is incorrect because these archaea cause methane production among mammals and particularly among humans, thus inhibiting research on these archaea in humans for a long time. Within my team and in collaboration with Michel Drancourt, we systematically began to look for archaea. This allowed us to significantly increase the number of known archaea in the commensal gut and to discover, for the first time, the presence of archaea in pathological samples (Drancourt, 2012). However, the microbial world was then still divided into three *domains* (Pace, 2006). Viruses were therefore excluded from microbes until recently. The steps of viral recognition initially included a filtration stage *using Chamberland filters* (*a coworker of Pasteur*). Indeed, viruses were originally defined by their invisibility in the light microscope (*infra-microbes was quoted by Pasteur*) and their persistence after filtration due to their small size. The steps of the discovery of viruses were as follows (Raoult, 2013): they were first identified as agents passing through a filter by Chamberland (Table 1); Pasteur then discovered the rabies virus, which was ultra-filterable and invisible (but Pasteur thought it was a microbe); and later Ivanosky and Beijeink worked on the tobacco mosaic virus (Bos, 1999; Enquist and Racaniello, 2013). The first animal virus was recognized by Loeffler (Enquist and Racaniello, 2013); then Francis D'Herelle discovered the bacteriophage, which is a virus that infects bacteria (d'Herelle, **1917**); later, Ruska first observed viruses using an electron microscope (Enquist and Racaniello, 2013). In 1935, while studying tobacco mosaic viruses, Stanley postulated that viruses were biological molecules and not microbes (Stanley, 1935). Temin later discovered retroviruses (Temin, 1995), and Torsvik and Dundas (1974) identified archaea viruses. Finally, in 2002, we first reported the existence of giant viruses among microbes (La Scola et al., 2003; Raoult et al., 2004).

The discovery of Mimivirus

While working on the collection of "isolated" bacteria grown in Amoeba, using the amplification of the gene common to all bacteria, 16S rDNA, we successfully identified a new species of *Legionella* and a new clade of *Chlamydia*. However, this collection contained a microbe that appeared to be a Gram-positive bacterium that we had not previously identified (Raoult et al., 2007). This is a key point in this story because it was a visible microorganism with no universal 16S or 18S rDNA, so it did not appear to be a eukaryote, bacteria or archaea. Electron microscopy allowed us to determine that it was probably a virus because of its typical icosahedral shape, which is not present except in the viral world. We began describing this virus, demonstrating that it had an eclipse phase that was concentrated in a virus-producing factory, and it was a virion containing DNA and RNA. It was as large as most of the intracellular bacteria we were working on at that time. The sequence of its genome, published in 2004, showed that it consisted of 1.2 megabases (Raoult et al., 2004), which was larger than many bacteria, especially the bacteria we were studying, and which we had sequenced the genome (*Rickettsia conorii* and *Tropheryma whipplei*). The discovery of Mimivirus shocked the virological community for some time; however, some authors thought it would be possible to neglect giant viruses and considered Mimivirus an exception.

The hunt for giant viruses

In collaboration with Bernard La Scola, we began to search for giant viruses. We were able to isolate over one hundred of these viruses using the amoeba *Acanthamoeba spp.* as *host*. The second family discovered was the Marseillevirus family, which shared multiple origins with the giant amoeba viruses (Boyer al., 2009). This point had also been raised by other authors showing the frequency of bacterial genes at the end of the Mimivirus genome (Filee and Chandler, 2012). Our study of Marseillevirus led us to reconsider the genomic structure and evolution of amoeba viruses. Indeed, most intracellular microorganisms, especially viruses, are facing only one carrier of genetic information within their ecosystem, their host itself. Under these conditions, the exchange of genes is extremely rare, and most of the time, only an exchange of genes with their host occurs. However, amebas are different beings. We have shown that they phagocytize all particles larger than 50 nm and thus bacteria, fungi, viruses, *and archaea can coexist in amoeba* (Raoult and Boyer, 2010). Gene flow can thus occur at a high level, which explains the chimeric appearance of all viruses isolated from amoebae. Thus, amoeba viruses, like these from other phagocytic protists (Slimani et al., 2013), are probably of a different nature *than other viruses*. This led us to believe that, on the one hand, they could be a source of new chimeric organisms; on the other hand, they also create a battleground for different organisms attempting to grow or survive within these protists. The discovery of Mimivirus and Marseillevirus has been followed by the identification of other viruses of the family Mimivirus, including *Megavirus chilensis* (Arslan et al., 2011), one of the three Mimiviride groups; Lausannevirus (Thomas et al., 2011) and Sénégalvirus (Lagier et al., 2012), which are viruses from the Marseillevirus family. It should be noted that the discovery of those giant viruses occurred in human samples; Marseillevirus was found in the blood (Popgeorgiev et al., 2013), lymph nodes (Popgeorgiev et al., 2013) and feces of humans (Lagier et al., 2012), while Mimivirus was found in respiratory samples and stool samples of patients with pneumonia (Saadi et al., 2013a, 2013b). The size of Mimivirus prevented these viruses from being discovered because when a particle was visible in the microscope, it was excluded from the viral world. Moreover, the assumed small size of viruses (below 0.2 μm) induced work that started with a filtration step, which retained giant viruses from the start, thus hindering their identification (Colson et al., 2013). Therefore, the viral definition based on a filtration step excluding particles larger

than 0.2 μm impeded the knowledge of giant viruses for over a century.

Redefining viruses

The discovery of Mimivirus has led us to attempt to reclassify viruses, which we accomplished with Patrick Forterre (Raoult and Forterre, 2008). Our conclusion was based on the fact that what united the genomes from archaea, bacteria and eukaryotes was a pool of genes associated with translation, in particular, ribosomal proteins, translation factors, transfer RNA synthetase amino acids, and chaperones. Furthermore, we found that transcription factors, particularly RNA polymerase, were DNA dependent. When the pool of genes common to all three domains was compared to giant viruses, the major difference was that those three domains all contained ribosomes and that the capsid and structural homologies revealed by Bamford in viruses distinguished them (Krupovic and Bamford, 2008). Based on this difference, we proposed a genomic classification that differentiated between organisms encoding ribosomes and organisms encoding the capsid; this classification did not include those that resisted the definitions, such as plasmids, retroposons, retroviruses, satellite DNA, transposons, viroids and those that were transmitted and contained only proteins, such as prions and nanons (Raoult and Forterre, 2008).

The new giant viruses

The door was then open for the research of giant viruses. In fact, one of our former co-workers who had helped us with the annotation of the genome sequence of Mimivirus, Jean-Michel Claverie and his wife C. Abergel, continued research into these new viruses. This work enabled them to discover two new viral families: Pithovirus (LeGendre et al., 2014) and Pandoravirus (Philippe et al., 2013). These viruses have questioned our classification based on the capsid of a virus because those viruses did not have a bona fide capsid and thus could not fit into this classification system. Our classification therefore survived only four years, which shows that our current theories should be modest because the evolution of knowledge forces us to constantly reassess previous classifications. A smaller related virus (*370 KB genome) was recently reported* (Moniruzzaman et al., 2014).

The discovery of the virophage

The virophage was discovered by chance with the isolation of the second strain of Mimivirus, which we *had* called Mamavirus (La Scola et al., 2008; Desnues et al., 2012; Yutin et al., 2013; Desnues and Raoult, 2010). A small virus was associated with this new strain that we first considered to be a satellite; Bernard La Scola named it Sputnik. Because it was the equivalent of a bacteriophage, I named it virophage. The reason I felt justified to call it a bacteriophage was that it presented all the elements of the cycle of a bacteriophage. It was an autonomous virus that encoded its capsid, had a specific phylogeny, was the size of an average a virus, slowed down the propagation of Mimivirus and created atypical forms of Mimivirus (Campos et al., 2014). It was not able to infect amoebae, and it multiplied on the outskirts *of the Mimivirus*. Finally, in some cases, the virophage could be packaged and could use the Mimivirus capsid as a "Trojan horse" to enter amoebae. By studying a form of Mimivirus, we showed that when Mimivirus multiplied alone in the amoeba, it lost 20% of its genes and the ability to create fibrils. *Fibrils are* essential to the virophage entrance and Mimivirus became virophage-resistant when the virophage lost its fibrils (Boyer et al., 2011). We studied and reported the virophage cycle, during which the virophage multiplies very early and intensely from the beginning of the formation of the virus factory. The presence of this virophage was reported by our team and later by another team in many samples from the environment as well as in many countries around the world (Fischer and Suttle, 2011). Other viruses have been isolated since then, including Sambabirus, which contained a virophage that once again demonstrated an inhibition of the multiplication of Mimivirus (Campos et al., 2014). This virophage seemed to have a very broad spectrum. This spectrum *allows* it to grow in the three groups comprising Mimivirus (group A with Mimivirus, group B and group C with Moumouvirus and *Megavirus chilensis*). This allowed us to use Mimivirus as a virophage reporter and to isolate virophages that had been molecularly detected in samples using the amoeba and Mimivirus to support its multiplication (Gaia et al., 2013). This discovery was immediately partly undermined by the fact that a new virophage, Zamilon, presented host specificity and seemed to be able to multiply only in members B and C of Mimivirides but not in group A (Gaia et al., 2014). In addition, we confirmed that the virophage did indeed have a cycle similar to that of bacteriophages because it had an integrated form within a mimirus (a provirophage) and could be associated with a transpoviron, the equivalent of a transposon that can be found in a virophage and is thus transmitted from Mimivirus to Mimivirus (Yutin et al., 2013). Therefore, a virophage can have the same cycle and functions as a bacteriophage by integrating itself as a provirus in lysogenizing Mimivirus and carrying at least one gene transpoviron of Mimivirus. This demonstrates that virophages are very different from satellites (Desnues and Raoult, 2012). The transpoviron also has an impressive multiplicative ability and appears to be the first gene expressed at such a level in amoebae that are infected by Mimivirus. Finally, the virophage seems to participate in the battle within the amoebae. Indeed, Mimivirus alone in this type of amoeba would not allow such a multiplication rate because the destruction of amoebae is so fast that it would completely destroy the population. We were able to demonstrate that the virophage was involved in the population control of Mimivirus and in intracellular bacteria, especially bacteria BABL1, where it decreases the multiplication rate of Mimivirus (Slimani et al., 2013). Thus, we must consider phagocytic protists to be a meeting place and a battlefield of different microorganisms that are highly complex and regulated in part by the interactions between these parasites.

News on virophages

Since our discovery of the virophage, other teams have been able to demonstrate that this phenomenon was not a unique phenomenon related to Mimivirus. Indeed, a virophage has been proposed as the origin of large transposons; the virophage was described as being associated with Mavirus and a parasite of *Cafeteria roenbergensis*, which encodes 20 proteins, including integrase (Fischer and Suttle, 2011). Furthermore, the Yau team proposed that the virophage participates in the control of algae in Antarctica and of their viral parasites. Thus, virophages are a new biological entity that certainly helps in regulating the populations of giant viruses (Yau et al., 2011). It is noteworthy that we were able to demonstrate the existence of seroconversion for both Mamavirus and the virophage in two patients returning from Laos with fever, suggesting that under certain conditions, the virophage can be a pathogen, and in all cases it is immunogenic, which was demonstrated by the fact that we found virus-specific proteins recognized by the serum of these patients (Parola et al., 2012). Therefore, the virophage indisputably raises the question of the very existence of satellites as entities because in reality they are not very homogeneous; they appear to overlap with the existence

of real viruses by presenting phage virions that contain proteins entirely encoded by its genome (Desnues and Raoult, 2012). *Conversely*, isolated sequences, such as the delta agent, correspond more strongly to nucleic *acid* parasites than to viruses. However, the debate remains open because it was not possible for us to understand the life cycle of a virophage by classifying it as a satellite, and the definition of satellites will certainly need to be reconsidered.

The virophage and the fourth "domain"

Upon the publication of the genome of Mimivirus, I suggested as a conclusion that this was a fourth domain of life, and I had to fight to keep this notion at the end of the article, despite the contrasting opinion of the editor and of my co-author Jean-Michel Claverie (Raoult et al., 2004). This concept was taken further by one of my students, Mohamed Amine Madaoui, who, by analyzing RNA polymerase that was dependent on DNA, obtained the same conclusion: there are four branches representing 4 different worlds, including eukaryotes, bacteria, *archaea and giant viruses* (Boyer et al., 2010). We should note that the tree of the transcription factor 2B is similar, and we found a genomic content in the giant viruses that differentiated them, in terms of information, from eukaryotes, archaea and bacteria, and constituted a world apart. This concept is highly controversial. However, the study of DNA-dependent RNA polymerases is very important, and this phylogenetic specificity has led to the discovery in databases of 3 giant viruses that had been completely ignored despite the fact that they represented a unique scaffold and *are* relatively easy to identify (Sharma et al., 2014). Two of these *genomes* have been *inadvertently* published in the journal Nature, one as an archaea and the other within the genome of a hydra. *Recognition and annotation* of giant viruses is *clearly* still in its infancy.

This led us to redefine the microbes as trucs (Raoult, 2013). Indeed, giant viruses have also been called Megavirales (Colson and Raoult, 2012), which have all the properties of microbes. They are parasites, but there are bacterial, eukaryotic or archaeal parasites as well. Giant viruses may be infected with parasite viruses themselves that contain transpovirons; these giant viruses have chimeric large genomes and are visible microscopically. Thus, the word "domain" has been created to accommodate the classification of microbes based on the ribosome (and almost exclusively on the ribosome) that Bill Martin called the Tree of 1% genes

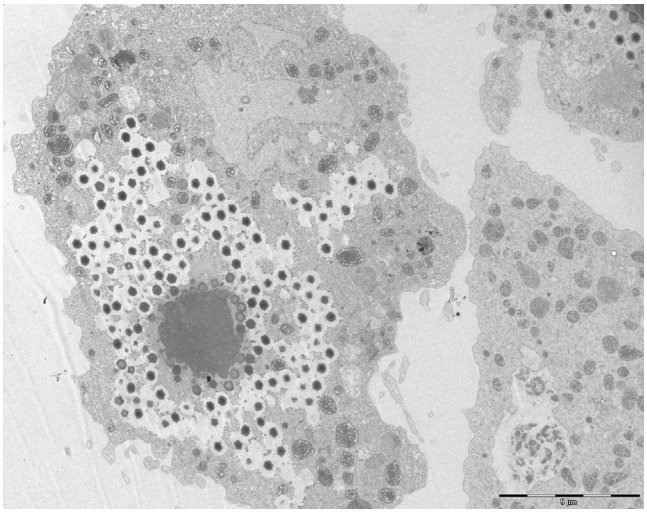

Fig. 2. Giant Longchamps virus (Mimiviridae group A) infecting the cytoplasm of the amoeba *Acanthamoeba polyphaga* after 14 h infection. Photo de Bernard La Scola (Angélique Campocasso et Audrey Borg).

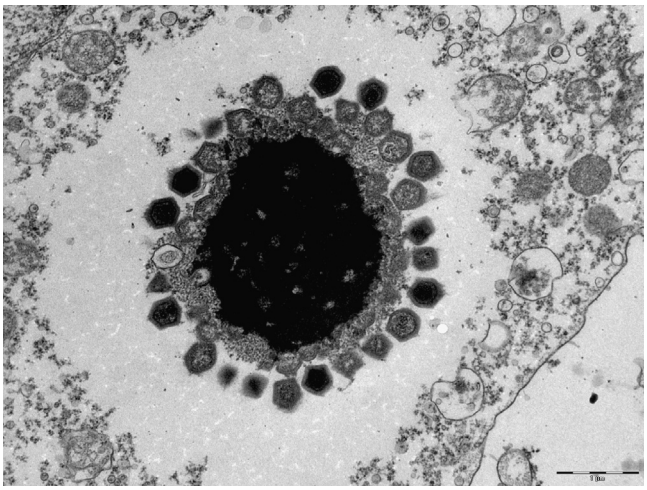

Fig. 3. Virus factory of Moumouvirus (Mimiviridae group B) in the cytoplasm of the amoeba *Acanthamoeba castellanii*, after 16 h infection. Photo de Marie Suzan (Bernard Campagna).

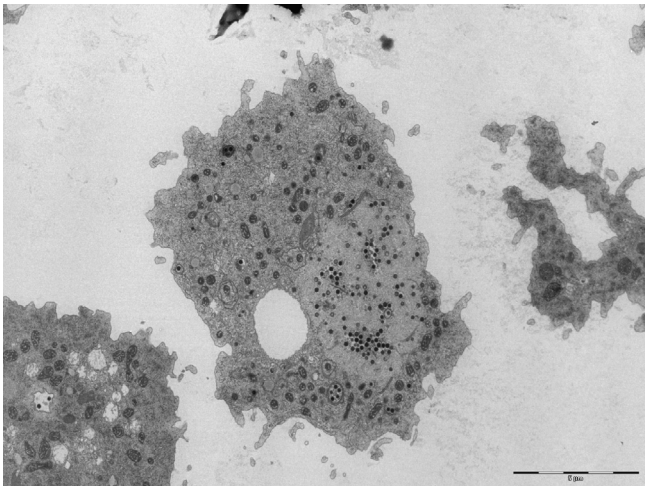

Fig. 1. Giant Marseillevirus (Marseilleviridae) infecting the cytoplasm of the amoeba *Acanthamoeba castellanii* after 12 h infection. Photo de Bernard La Scola (Isabelle Pagnier, Audrey Borg).

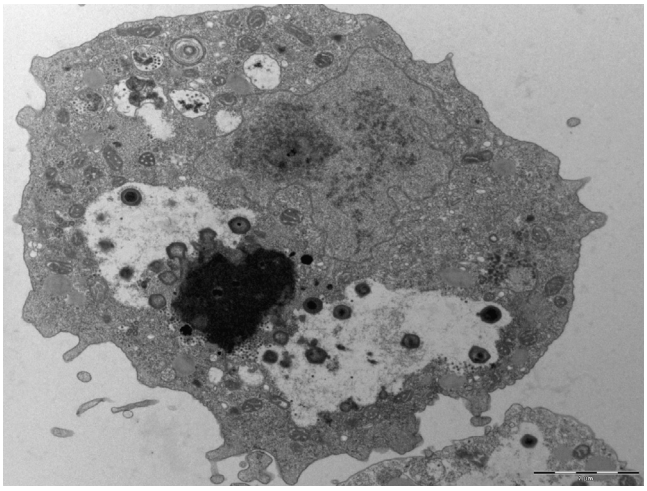

Fig. 4. Infection of the amoeba *Acanthamoeba castellanii* with both giant virus Mamavirus (Mimiviridae Group A) and the virophage Sputnik 1, after 16 h infection. Photo de Bernard La Scola (Bernard Campagna).

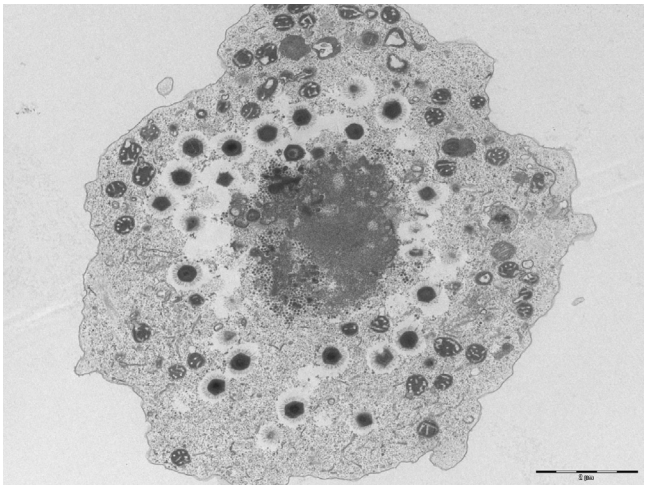

Fig. 5. Infection of the amoeba *Acanthamoeba polyphaga* with both giant virus Mont1 (Mimiviridae Group C) and the virophage Zamilon, after 16 h infection. Photo de Bernard La Scola (Mondher Boughalmi, Audrey Borg).

(Dagan et al., 2010). It was necessary to create a new name to define the 4 worlds constituting the microbes; I chose the term "truc" (meaning "stuff" in French) to include bacteria, eukaryotes, archaea and Megavirales. It is important to note that one should not consider these microbes as homogeneous or as reflecting evolution as described by Darwin. Each of them is a mosaic of genes from different origins; some contain genes of archaic origin, others have been derived from exchanges of recent sequences, and others probably came from genes created a new, especially from the RNA world. The tree of life exists only as a representation, *a fantasy* that we will not be able *to* maintain in the XXIst century, and it may be replaced by the rhizome of life (Raoult, 2010). However, the morphological and functional definitions of organisms are perfectly valid, and that is why I prefer the term "truc" to classify what we see as microbes Figs. 1–5.

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